# Growth, Nitrogen Fixation, and Nodularin Production by Two Baltic Sea Cyanobacteria

JAANA LEHTIMÄKI,[1] PIA MOISANDER,[2]† KAARINA SIVONEN,[1]* AND KAISA KONONEN[2]

*Department of Applied Chemistry and Microbiology, Division of Microbiology, Biocenter Viikki,
FIN-00014 Helsinki University,[1] and Finnish Institute of Marine Research, FIN-00931 Helsinki,[2] Finland*

In late summer, nitrogen-fixing cyanobacteria *Nodularia spumigena* and *Aphanizomenon flos-aquae* form blooms in the open Baltic Sea. *N. spumigena* has caused several animal poisonings, but Baltic *A. flos-aquae* is not known to be toxic. In this laboratory study, performed with batch cultures, the influences of environmental conditions on the biomass and nitrogen fixation rate of *N. spumigena* and *A. flos-aquae* were compared and the toxin (nodularin) concentration produced by *N. spumigena* was measured. Several differences in the biomasses and nitrogen fixation rates of *N. spumigena* and *A. flos-aquae* were observed. *A. flos-aquae* preferred lower irradiances, salinities, and temperatures than *N. spumigena*. The biomass of both species increased with high phosphate concentrations and with accompanying bacteria and decreased with unnaturally high inorganic nitrogen concentrations. Nodularin concentrations in cells and growth media, as well as nitrogen fixation rates, were generally highest under the conditions that promoted growth. Intracellular nodularin concentrations increased with high temperature, high irradiance, and high phosphate concentration and decreased with low and high salinities and high inorganic nitrogen concentrations. Nodularin concentrations in growth media increased with incubation time, indicating that intracellular nodularin was released when cells lysed. The different responses of *A. flos-aquae* and *N. spumigena* to changes in salinity, irradiance, and temperature may explain the different spatial and temporal distribution of these species in the Baltic Sea. According to the results, toxic *N. spumigena* blooms may be expected in late summer in areas of the Baltic Sea with high phosphorus concentrations and moderate salinity.

Late-summer blooms of heterocystous cyanobacteria are common in the Baltic Sea, the largest brackish water basin in the world. Based on the satellite data, it has been suggested that cyanobacterial blooms have increasingly expanded there into new areas (13). *Nodularia spumigena* and *Aphanizomenon flos-aquae* are the dominant cyanobacterial species found in the open Baltic Sea. *A. flos-aquae* is not known to be toxic (36), but several blooms of *N. spumigena* have been associated with the poisonings of animals (for a review, see reference 24).

*N. spumigena* produces nodularin, a cyclic pentapeptide hepatotoxin with the structure of cyclo(-D-*erythro*-β-methyl-Asp-L-Arg-Adda-D-Glu-*N*-methyldehydro-β-aminobutyric acid), in which Adda is (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (29, 36). A similar cyclic pentapeptide, motuporin, has been recently isolated from a marine sponge, *Theonella swinhoei*, in which the compound is probably produced by a symbiotic cyanobacterium (6). Three new minor variants of nodularin have been characterized from a bloom and a culture of *N. spumigena* from New Zealand (23). During the study of the biosynthesis of nodularin, linear nontoxic peptides, thought to be precursors of nodularin, have been isolated from cultured *N. spumigena* (30).

Cyanobacterial hepatotoxins such as nodularin inhibit serine/threonine-specific protein phosphatases 1 and 2A, which leads to the disruption of the structure and function of intermediate filaments and microfilaments in the liver (4). These inhibitors of protein phosphatases can accelerate tumor development in nonlethal doses (4). In acute doses, the animals die of hemorrhagic shock in a few hours (4). The acute toxicity of nodularin is 70 µg kg$^{-1}$ (intraperitoneally in mouse) (33). In blooms, the concentration of nodularin often rises high enough to cause a health risk for animals and humans. However, the role of nodularin for *N. spumigena* or its effect on water environments is not known yet. Hepatotoxic *N. spumigena* blooms have been observed in all areas of the Baltic Sea except in the most northern part of the Gulf of Bothnia, the Bothnian Bay (15, 36).

An important reason why *N. spumigena* and *A. flos-aquae* are capable of forming blooms in the nitrogen-depleted water mass of the Baltic Sea in the late summer is their ability to fix dinitrogen. However, these species differ from each other in ecology, demonstrated by the fact that *N. spumigena* is absent from the surface waters during most of the year, while *A. flos-aquae* is found there during the whole year (reference 25 and references therein). Also, the vertical and horizontal distributions of these two species differ in the Baltic Sea (18, 25). In order to obtain information of the codominance of these species in the late summer blooms, we used batch culture experiments to study the effects of a number of environmental factors on biomasses, nitrogen fixation rates, and nodularin concentrations. A knowledge of the physiological responses of these species is essential for understanding cyanobacterial bloom dynamics in the Baltic Sea. This study was facilitated by the successful isolation of the Baltic *A. flos-aquae* strain and the purification of axenic clones of Baltic *A. flos-aquae* and *N. spumigena*.

* Corresponding author. Mailing address: Department of Applied Chemistry and Microbiology, Division of Microbiology, P.O. Box 56, Biocenter Viikki, FIN-00014 Helsinki University, Finland. Phone: 358-9-70859270. Fax: 358-9-70859322. E-mail: Kaarina.Sivonen@Helsinki.Fi.

† Present address: Institute of Marine Sciences, University of North Carolina at Chapel Hill, 3431 Arendell Street, Morehead City, NC 28557.

## MATERIALS AND METHODS

**Organisms.** Hepatotoxic *Nodularia spumigena* BY1 (36, 37) and nontoxic *Aphanizomenon flos-aquae* TR183 (this study) were isolated from blooms in the

TABLE 1. Growth conditions used in experiments with *A. flos-aquae* and *N. spumigena* strains from the Baltic Sea

| Expt | Condition(s) | | | | | |
|---|---|---|---|---|---|---|
| | Accompanying bacteria | Temp (°C) | Light flux ($\mu$mol m$^{-2}$ s$^{-1}$) | Salinity (‰) | PO$_4$-P ($\mu$g liter$^{-1}$) | NH$_4$/NO$_3$-N ($\mu$g liter$^{-1}$) |
| Accompanying bacteria | ± | 20 | 25 | 5 | 5,500 | 0 |
| Temp | − | 7, 10, 13, 16, 19, 22, 25, 28 | 25 | 5 | 5,500 | 0 |
| Light flux | − | 20 | 2, 25, 45, 105, 115, 155 | 5 | 5,500 | 0 |
| Salinity | + | 20 | 25 | 0, 5, 10, 15, 20, 30 | 5,500 | 0 |
| Phosphorus concn | − | 20 | 25 | 5 | 0, 0.2, 2, 20, 200, 1,700, 5,500 | 0 |
| Nitrogen concn and source | − | 20 | 25 | 5 | 5,500 | 0, 20, 200, 2,000, 42,000 |

Arkona Sea (55°00.00′N, 13°18.00′E), and the Gulf of Finland (59°12.85′N, 22°02.5′E). The isolation of the *A. flos-aquae* strain was done as described before for *N. spumigena* (37), except that the salt concentration of the growth medium was lowered (0 to 5‰). Axenic cultures of both strains were obtained by the soft agarose-plating method (32).

**Batch experiments.** The effects of accompanying bacteria, temperature, light flux, salinity, phosphate, and different inorganic nitrogen sources and concentrations were studied in separate experiments (Table 1). Responses in biomasses (chlorophyll *a*, dry weight, and total protein), nitrogen fixation rates, and toxin concentrations were monitored with four, three, and two replicates, respectively. The effects were observed for 21 days, except in the phosphorus experiment where *N. spumigena* was grown for 43 days, due to slow growth (dry weight and nitrogen fixation rate were not measured on day 43). The experiments were done in batch cultures in temperature-controlled water baths under continuous illumination as described earlier (17). The growth medium was Z8 with salt added and nitrogen omitted (reference 37 and references therein), except in experiments where the salinity was changed by adding different amounts of a mixture of NaCl (87.5 g liter$^{-1}$) and MgSO$_4$ · 7H$_2$O (37.5 g liter$^{-1}$) or where the concentration and source of nitrogen were modified by adding an appropriate amount of NaNO$_3$ or NH$_4$Cl. In the phosphorus experiment, the concentration of phosphate was reduced by replacing some or all of K$_2$HPO$_4$ · 3H$_2$O with KCl in order to obtain the desired experimental phosphorus concentrations. Inocula were grown at a temperature of 20°C and a light flux of 25 $\mu$mol m$^{-2}$ s$^{-1}$ in the modified Z8 medium for 7 days. In the phosphorus experiment the inocula were grown without phosphate to deplete cellular phosphorus reserves. The inoculum was 5 ml per 100 ml of growth medium.

**Analyses.** Samples were taken six times from the cultures during the study period, except in the phosphorus experiment, where *N. spumigena* was sampled seven times. In all experiments, culture purity was checked on TGY (tryptone-glucose-yeast extract) plates which were incubated at 28°C for 5 days (1). In the bacterium experiment, bacterial numbers were counted by using the acridine orange method (8), and bacterial production was measured by thymidine incorporation (2). In preliminary experiments, 50 nM thymidine (specific activity, 5 Ci mmol$^{-1}$; Amersham) was found to saturate the thymidine uptake of the nonaxenic *N. spumigena* culture, but the thymidine uptake increased until the thymidine concentration was as high as 200 nM in the nonaxenic *A. flos-aquae* culture (data not shown). However, 50 nM methyl-$^3$H-thymidine was used for experiments with both strains. After the incubation of a 5-ml sample under the same light and temperature conditions as during the whole study period, 100 $\mu$l of formalin was added to the samples. The samples were then filtered onto cellulose nitrate filters (Sartorius) and rinsed ca. 9 or 10 times with 1 ml of ice-cold 5% trichloroacetic acid. After addition of 10 ml of scintillate (Instagel), the radioactivity on the filters was measured with a Wallac 1411-001 scintillation counter (Turku, Finland). Three different biomass parameters were measured. Dry weights and chlorophyll *a* concentrations were determined as previously described (17, 38). Total protein contents were determined at 500 nm by the method of Lowry with bovine serum albumin as a standard (7). Nitrogen fixation rates were measured by the acetylene reduction technique as described earlier (22). The rates per volume were normalized to the concentration of chlorophyll *a* to obtain the nitrogenase activities per biomass unit. The cyanobacterium samples were fixed with Lugol's solution, and the filament length and heterocyst frequency were measured for at least 50 filaments from each sample by using light microscopy. Nodularin from the cells was analyzed by high-performance liquid chromatography (Hewlett Packard 1090M chromatograph) with an internal surface reverse-phase column (Pinkerton GFF-S5-80; inner diameter, 150 by 4.6 mm; Regis Chemical Company) and pure nodularin as a quantitative standard (17, 21). Nodularin concentrations from growth media were analyzed as cell samples after the nodularin was concentrated by using C$_{18}$ octadecyl cartridges

(Bond Elut; Varian) as previously described (17). Intra- and extracellular toxin values were normalized to measurements of dry weight and chlorophyll *a*, respectively.

**Statistical analyses.** The relatedness of different parameters was tested with correlations (SPSS for Windows 6.0, 1993, and Matlab for Windows 3.1, 1994). Because of a large number of zero values, the chlorophyll *a* concentration, nitrogen fixation rate, and intracellular toxin concentration data were not normally distributed (normality tested by Lilliefors test). Therefore, nonparametric tests (Spearman and Kendall rank correlation coefficients) were used to evaluate the degree of correlations among chlorophyll *a* concentrations, nitrogen fixation rates, and intracellular toxin concentrations (SPSS). In order to compare the different methods of biomass measurement, total protein, chlorophyll *a*, and dry weight data were ln-transformed before using the parametric test (Pearson correlation coefficient). Multiple regression analysis (Matlab) was performed for chlorophyll *a* data for each experiment except for the experiment with accompanying bacteria. First, a transformation between original and coded variables was computed; then, interaction terms of test variables were appended to matrices.

## RESULTS

In general, the biomass parameters (chlorophyll *a*, dry weight, total protein) were significantly correlated in the different experiments. In the salinity experiment, dry weight and salinity had a positive correlation ($P < 0.001$) indicating that the filters used for dry weight determinations retained salt; thus, dry weight was not valid for estimating growth in the salinity experiment.

Biomass (chlorophyll *a*) and normalized nitrogen fixation rates of both strains correlated positively ($P < 0.05$) in NO$_3$-N experiments. The same was true for *A. flos-aquae* in the phosphorus experiment ($P < 0.001$). On the other hand, nitrogen fixation rates per volume and biomass correlated positively ($P < 0.01$) in all experiments, except for *A. flos-aquae* in the bacterium experiment. Also, biomass and intracellular toxin correlated positively ($P < 0.005$) in all experiments (not examined in nitrogen experiments). Due to the poor growth of *N. spumigena* during the first 21 days of incubation in the phosphorus experiment, the correlation test was made only for the data from day 43. The test showed that chlorophyll *a* correlated positively with intra- and extracellular toxin concentrations ($P < 0.01$).

The normalized nitrogen fixation rates [in millimoles of ethylene (gram of chlorophyll *a*)$^{-1}$ hour$^{-1}$] were generally highest during the first week of the experiment and then decreased. Nitrogen fixation was often highest in conditions which promoted the growth. However, *A. flos-aquae* showed different responses for nitrogen fixation rates and biomass in temperature, light, and phosphorus experiments, and both strains showed different responses in the bacterium experiment. The

frequency of heterocysts and normalized nitrogen fixation rate of *A. flos-aquae* were positively related in bacterium ($P < 0.05$), temperature ($P < 0.01$), phosphorus ($P < 0.05$), and nitrate ($P < 0.001$) experiments (not determined for the light experiment). Positive relationships between the frequency of heterocysts and nitrogen fixation rate of *N. spumigena* were found in salinity ($P < 0.05$) and ammonium ($P < 0.01$) experiments (not determined for light and phosphorus experiments). Although the heterocyst frequency of *A. flos-aquae* was lower (ca. 10 mm$^{-1}$) than that of *N. spumigena* (30 to 40 mm$^{-1}$) (data not shown), the nitrogen fixation rates were higher in *A. flos-aquae* than in *N. spumigena*. The filament length and heterocyst frequency of *A. flos-aquae* were positively related in temperature ($P < 0.01$), salinity ($P < 0.01$), ammonium ($P < 0.001$), and nitrate ($P < 0.01$) experiments (not determined for the light experiment). A positive relationship between the filament length and heterocyst frequency of *N. spumigena* was found only in the salinity experiment ($P < 0.05$) (not determined for light and phosphorus experiments).

**Accompanying bacteria.** The number of heterotrophic bacteria (Fig. 1a) and the bacterial production (Fig. 1b) of both nonaxenic cultures increased during the study period. No thymidine incorporation was detected in the axenic strains. The nonaxenic *N. spumigena* and *A. flos-aquae* cultures grew better than the axenic ones (Fig. 1c), but no clear differences between axenic and nonaxenic cultures in nitrogen fixation rates (Fig. 1d) or intra- and extracellular toxin concentrations (Fig. 1e and f) were noticed. In the nonaxenic cultures, the filaments were longer and the heterocyst frequencies were higher than in the axenic cultures (data not shown).

**Temperature.** The growth of *N. spumigena* was low during the first 11 days, after which the strain grew best at the highest temperatures studied (25 to 28°C), while *A. flos-aquae* grew best at 16 to 22°C (Fig. 2a and b). A rise in the temperature increased the biomass of *N. spumigena* (positive linear regression coefficient, $P < 0.001$). During the time course, the biomass of *N. spumigena* decreased at low temperature and increased at high temperature ($P < 0.001$, positive regression coefficient for the coeffect). For *A. flos-aquae* the highest nitrogen fixation rates were seen at lower temperatures than the optimum growth temperature (Fig. 2c), but nitrogen fixation rates of *N. spumigena* were highest in the cultures that grew best (Fig. 2d). At temperatures of 7, 10, and 16°C, biomass remained too low for nodularin measurements (Fig. 2e). Extracellular toxin concentrations were highest at the highest temperatures studied (Fig. 2f).

**Light flux.** *A. flos-aquae* grew best at low irradiances (25 to 45 µmol m$^{-2}$ s$^{-1}$) (Fig. 3a), but highest nitrogen fixation rates were found from cultures grown at high irradiances (Fig. 3c). *N. spumigena* preferred higher irradiances (45 to 155 µmol m$^{-2}$ s$^{-1}$) (Fig. 3b and d). The positive impact of high light on the biomass of *N. spumigena* was also shown by the positive linear regression coefficient of light ($P < 0.001$). Light and time had a coeffect on the biomass of *N. spumigena* (positive correlation coefficient, $P < 0.01$): during the time course the biomass decreased at low light and increased at high light. Intra- and extracellular toxin concentrations were highest at the highest irradiances (Fig. 3e and f).

**Salinity.** *A. flos-aquae* tolerated salinities from freshwater to 10‰ (Fig. 4a and c). The significant negative linear regression coefficient of salinity ($P < 0.001$) showed the negative impact of high salinities on the biomass of *A. flos-aquae*. The biomass of *A. flos-aquae* increased at low salinity and decreased at high salinity during the time course (negative regression coefficient for the coeffect, $P < 0.001$). The nitrogen fixation rates of *A. flos-aquae* were similar in salinities of 0, 5, and 10‰ (Fig. 4c),

but the growth in 0‰ and 5‰ was much faster than in 10‰ (Fig. 4a). The biomass of *N. spumigena* was higher at moderate salinities (5 to 20‰) than at low (0‰) or high (30‰) ones (Fig. 4b). Also, the negative regression coefficient for the quadratic effect of salinity ($P < 0.001$) showed the decreasing effect of low and high salinities on the biomass of *N. spumigena*. The nitrogen fixation rate of *N. spumigena* was first promoted by the highest salinity, but later the nitrogen fixation rate was lowest in the highest salinity and highest at moderate salinities (Fig. 4d). The *N. spumigena* cells which grew best contained the highest toxin concentrations (Fig. 4e). The highest extracellular toxin concentrations were found at salinities of 5 to 10‰ (Fig. 4f).

**Phosphorus.** Phosphate concentrations of 200 µg liter$^{-1}$ or higher promoted the growth of *A. flos-aquae* (Fig. 5a). The nitrogen fixation rate of *A. flos-aquae* was also first increased by high phosphorus, but later no differences between the phosphorus concentrations were seen (Fig. 5c). Heterocyst frequency was clearly highest in the two highest phosphorus enrichments, implying high nitrogen fixation activity (data not shown). The growth of *N. spumigena* was low during the first 21 days (Fig. 5b). The nitrogen fixation rate of *N. spumigena* was barely detectable during the first 21 days (data not shown). On day 43 the biomasses (Fig. 5b) and the toxin concentrations (Fig. 5d and e) were high in the phosphorus-rich media and low under phosphate limitation.

**Nitrogen.** Cultures showed no differences in biomasses, nitrogen fixation rates, or nodularin concentrations in NO$_3$-N concentrations of 0 to 2,000 µg liter$^{-1}$ (data not shown). The same was true for *A. flos-aquae* in similar NH$_4$-N concentrations (data not shown). The highest nitrogen concentration used (42,000 µg liter$^{-1}$) decreased growth, nitrogen fixation rate, and nodularin production and lowered the heterocyst frequency (data not shown). The negative regression coefficient for the coeffect of time and ammonium ($P < 0.05$) suggested that during the time course, the biomass of *N. spumigena* increased under low extracellular NH$_4$-N concentrations and decreased at high concentrations. Also, ammonium itself was shown to have a significant negative effect on the biomass of *N. spumigena* (linear regression coefficient, $P < 0.01$).

## DISCUSSION

In the Baltic Sea, *N. spumigena* and *A. flos-aquae* show different patterns in their horizontal distribution. With the increasing salinity from freshwater in the north to approximately 15‰ salinity in the southern Baltic proper, the abundance of *A. flos-aquae* decreases while the abundance of *N. spumigena* increases (25). In the northern part of the Gulf of Bothnia where salinity approaches freshwater, *N. spumigena* is absent. The growth and nitrogen fixation rates of our *N. spumigena* isolate were highest in the same salinity range (5 to 20‰) in which the species forms mass occurrences in the Baltic Sea and other brackish waters (for a review, see reference 14). The incapability of *A. flos-aquae* to tolerate salinities higher than 10‰ suggests that salinity is an important factor limiting the distribution of this species.

The effects of temperature and light are seen in the seasonal and vertical abundance of *N. spumigena* and *A. flos-aquae*. The occurrence of *N. spumigena* blooms is restricted to summer months when the water temperature reaches about 15°C and the water mass is vertically stable (10, 14). *A. flos-aquae* is abundant in the water mass during the whole year, thus showing the ability to grow in low temperatures (25), which was also shown by this study. Vertically, *A. flos-aquae* is more homoge-
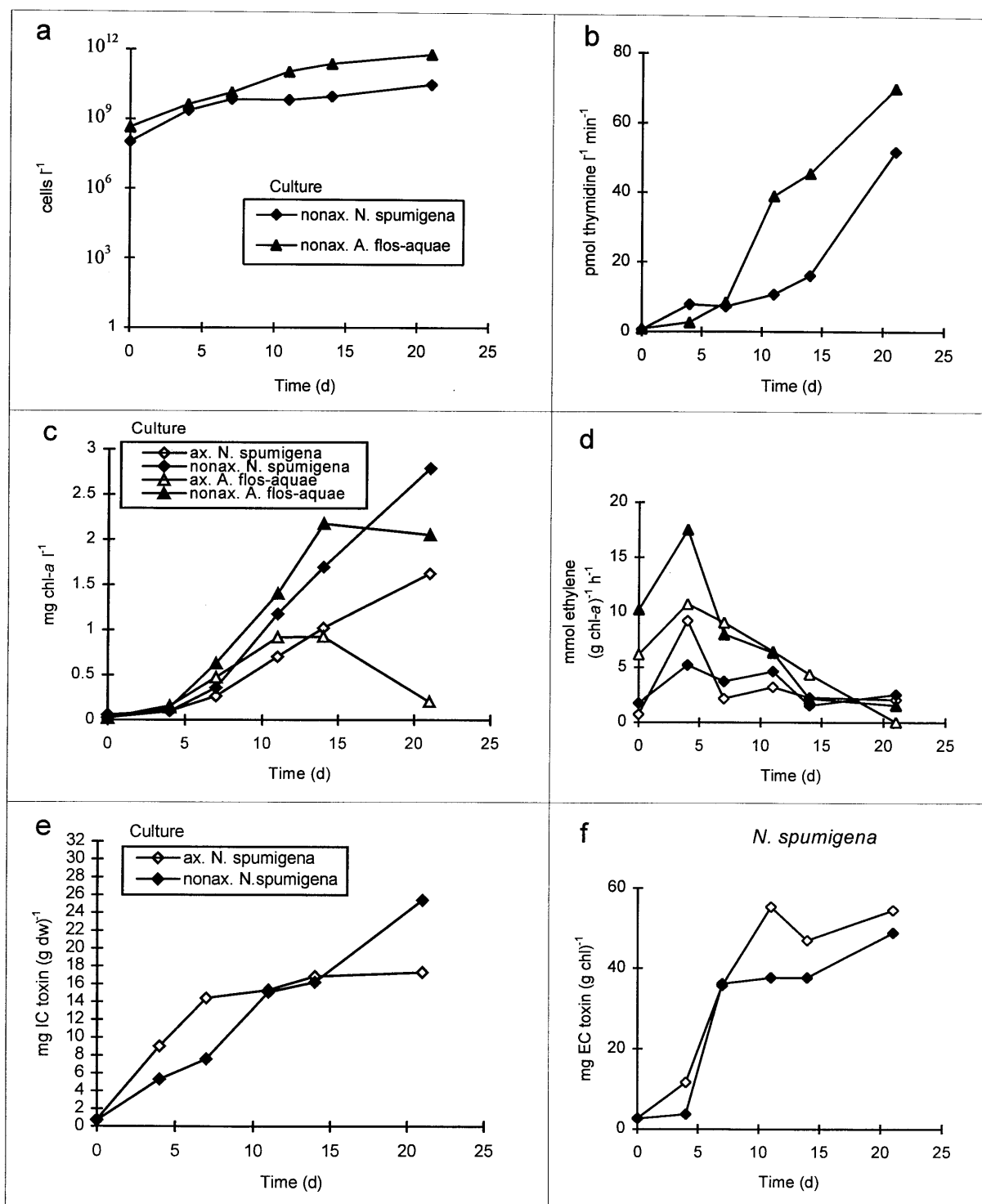
FIG. 1. Number (a) and production (b) of accompanying bacteria in nonaxenic (nonax.) cultures, and the effect of axenity on the biomass (c) and nitrogen fixation rate (d) of *A. flos-aquae* and *N. spumigena* cultures, and on intracellular (IC) (e) and extracellular (EC) (f) toxin concentrations. Percent coefficient of variation was generally $\leq 20$ in panels b, c, and e; $\leq 40$ in panel d; and $\leq 30$ in panel f, but in the cultures with poor growth it increased.

neously distributed than *N. spumigena*, which usually occurs only in the upper mixed layer and forms scums onto the water surface. In this study, *N. spumigena* grew fastest at temperatures of 25 to 28°C, showing a capacity to tolerate much higher temperatures than it experiences in its natural environment. Its

growth rate was slower at temperatures below 16°C than at higher temperatures, which has been also observed in the field (10, 14). The preference of *A. flos-aquae* for low light flux and that of *N. spumigena* for high light mirrored their vertical distribution patterns in the field. The growth and nitrogen
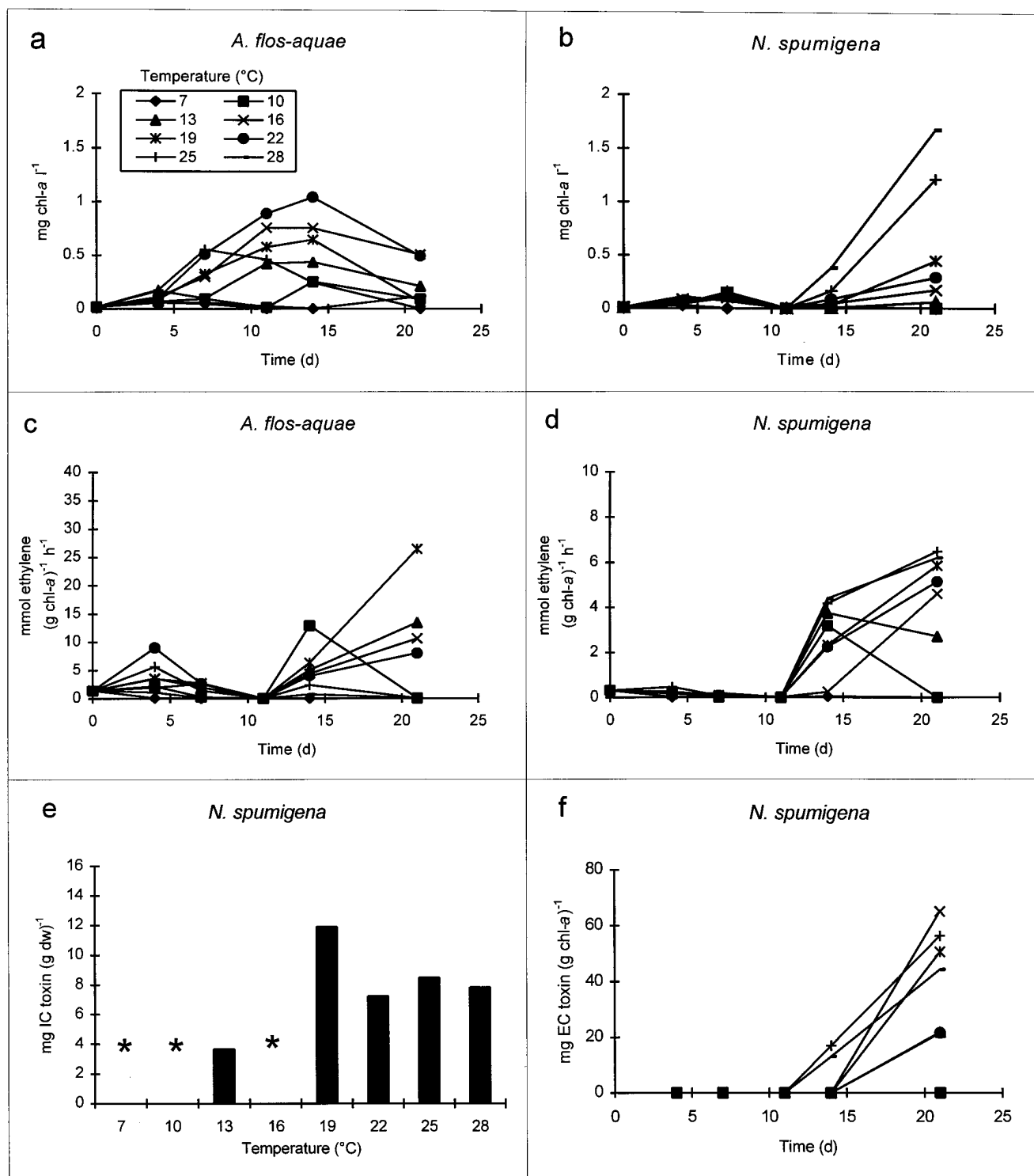
FIG. 2. Effect of temperature on the biomass (a and b) and nitrogen fixation rate (c and d) of *A. flos-aquae* and *N. spumigena* cultures and on intracellular (IC) (average values during the time course) (e) and extracellular (EC) (f) toxin concentrations. Percent coefficient of variation was generally ≤40 in panels a to d and ≤30 in panel f, but in the cultures with poor growth it increased. *, missing samples.

fixation rates of *N. spumigena* were stimulated by highest irradiances tested.

High phosphorus concentration and low N:P-ratio promote mass occurrences of *A. flos-aquae* and *N. spumigena* in the Baltic Sea (10, 14). Also, phosphorus enrichment studies have been shown to stimulate growth of Baltic Sea cyanobacteria (20, 31). The slow growth of *N. spumigena* during the phosphorus experiment in this study was probably due to phospho-
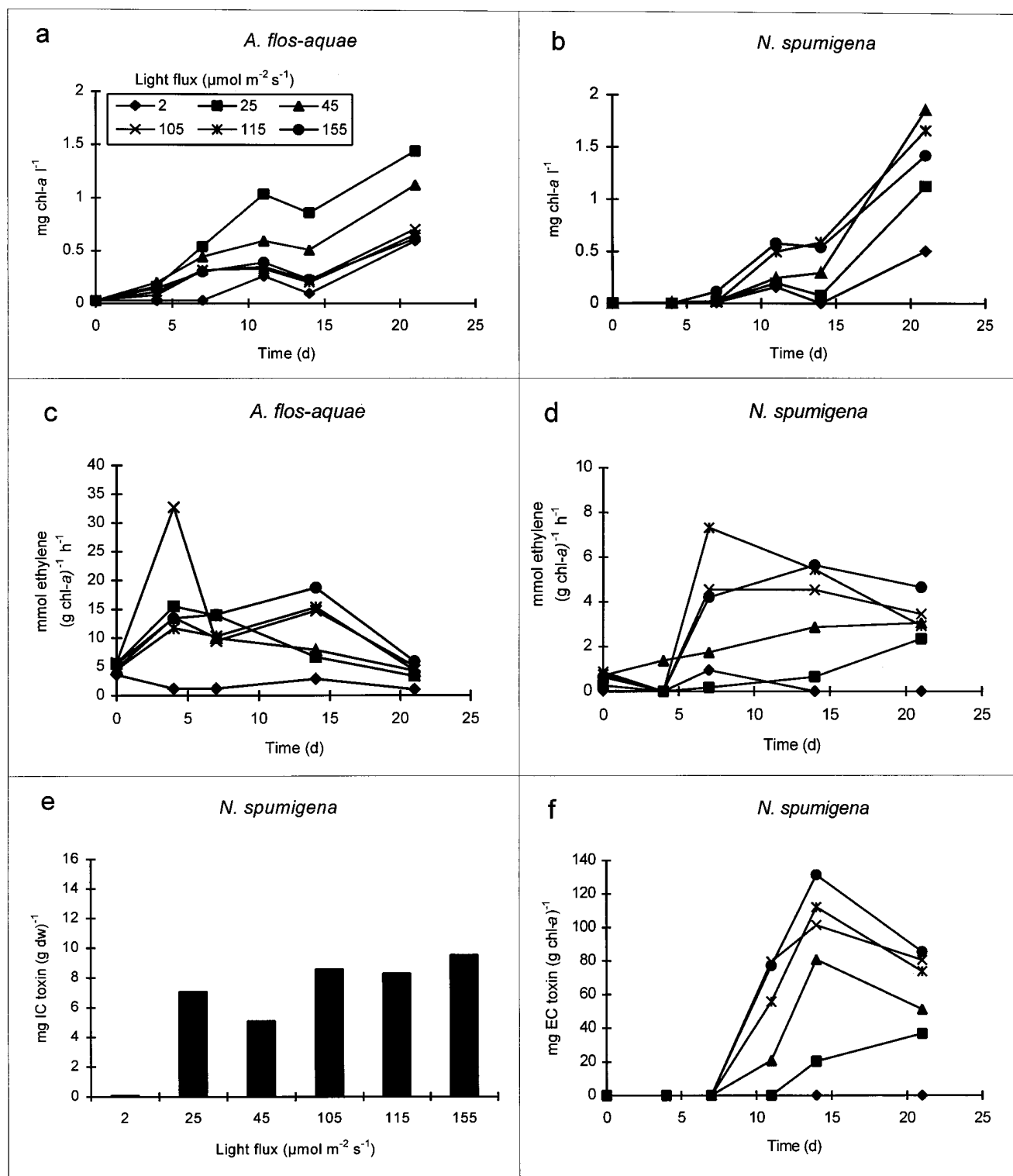
FIG. 3. Effect of light flux on the biomass (a and b) and nitrogen fixation rate (c and d) of *A. flos-aquae* and *N. spumigena* cultures and on intracellular (IC) (average values during the time course) (e) and extracellular (EC) (f) toxin concentrations. Percent coefficient of variation was generally $\leq 20$ in panels a and b, $\leq 40$ in panels c and d, and $\leq 30$ in panel f, but in the cultures with poor growth it increased.

rus starvation of the inoculum. The phosphorus starvation did not affect the biomass of *A. flos-aquae*, which may be due to its higher cellular phosphorus pools or lower phosphorus demand compared to *N. spumigena*. The effective utilization of high

phosphorus concentration by *A. flos-aquae* seems to be its strategy to form blooms in frontal and upwelling regions in the Baltic Sea (reference 14 and references therein), but the differences in phosphorus uptake kinetics between *A. flos-aquae*
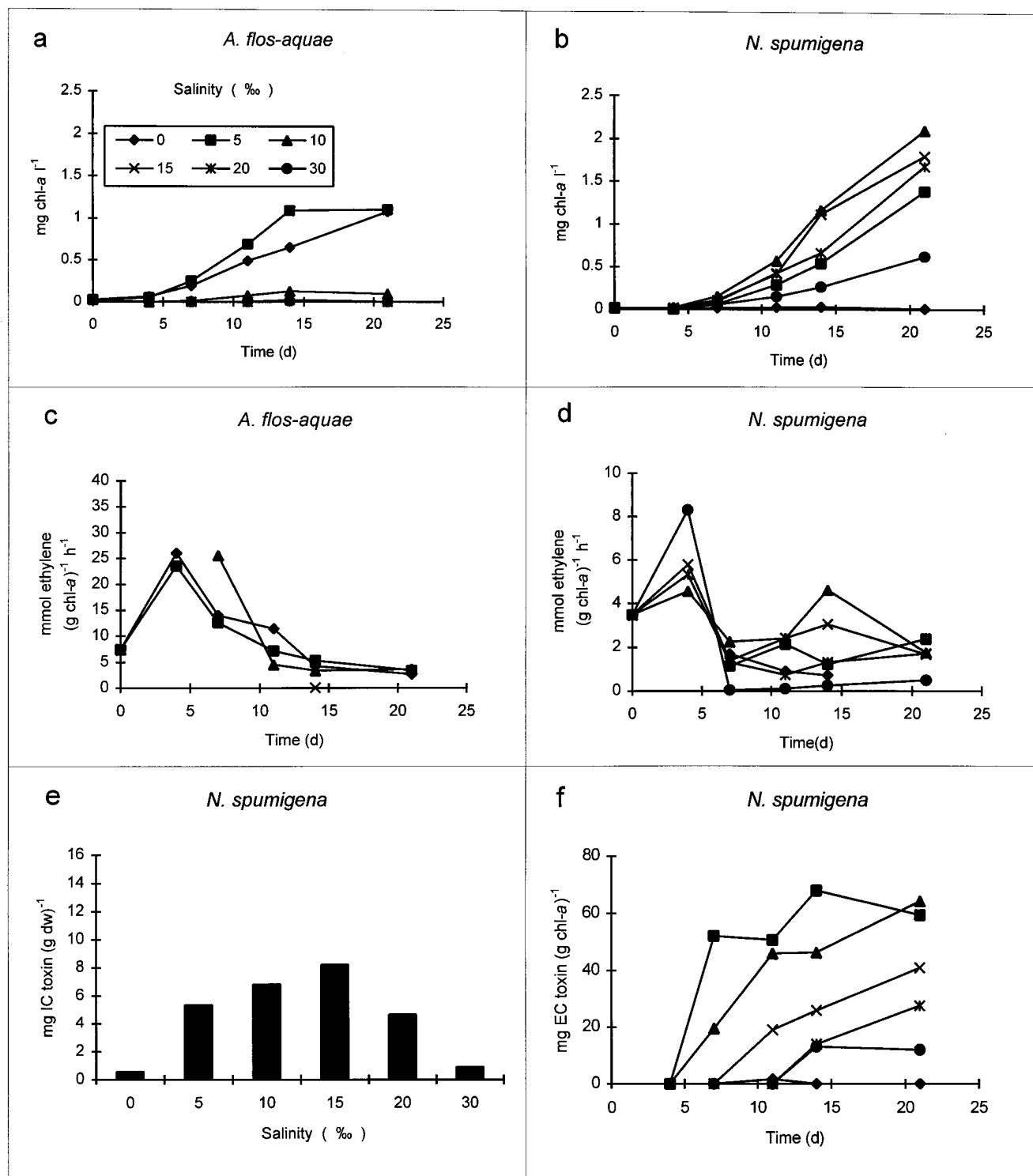
FIG. 4. Effect of salinity on the biomass (a and b) and nitrogen fixation rate (c and d) of *A. flos-aquae* and *N. spumigena* cultures and on intracellular (IC) (average values during the time course) (e) and extracellular (EC) (f) toxin concentrations. Percent coefficient of variation was generally ≤30 in panels a and b and ≤40 in panels c, d, and f, but in the cultures with poor growth it increased.

and *N. spumigena* and their abilities to store phosphorus require future studies.

Even low ammonium concentrations have been related to disappearance of heterocysts and nitrogen fixation of *N. spumigena* (11, 34). In this study, a decrease in biomass of *N.*

*spumigena* was also seen when ammonium was present. The highest nitrogen concentrations used in this study were detrimental to the growth and nitrogen fixation of *A. flos-aquae* and *N. spumigena.*

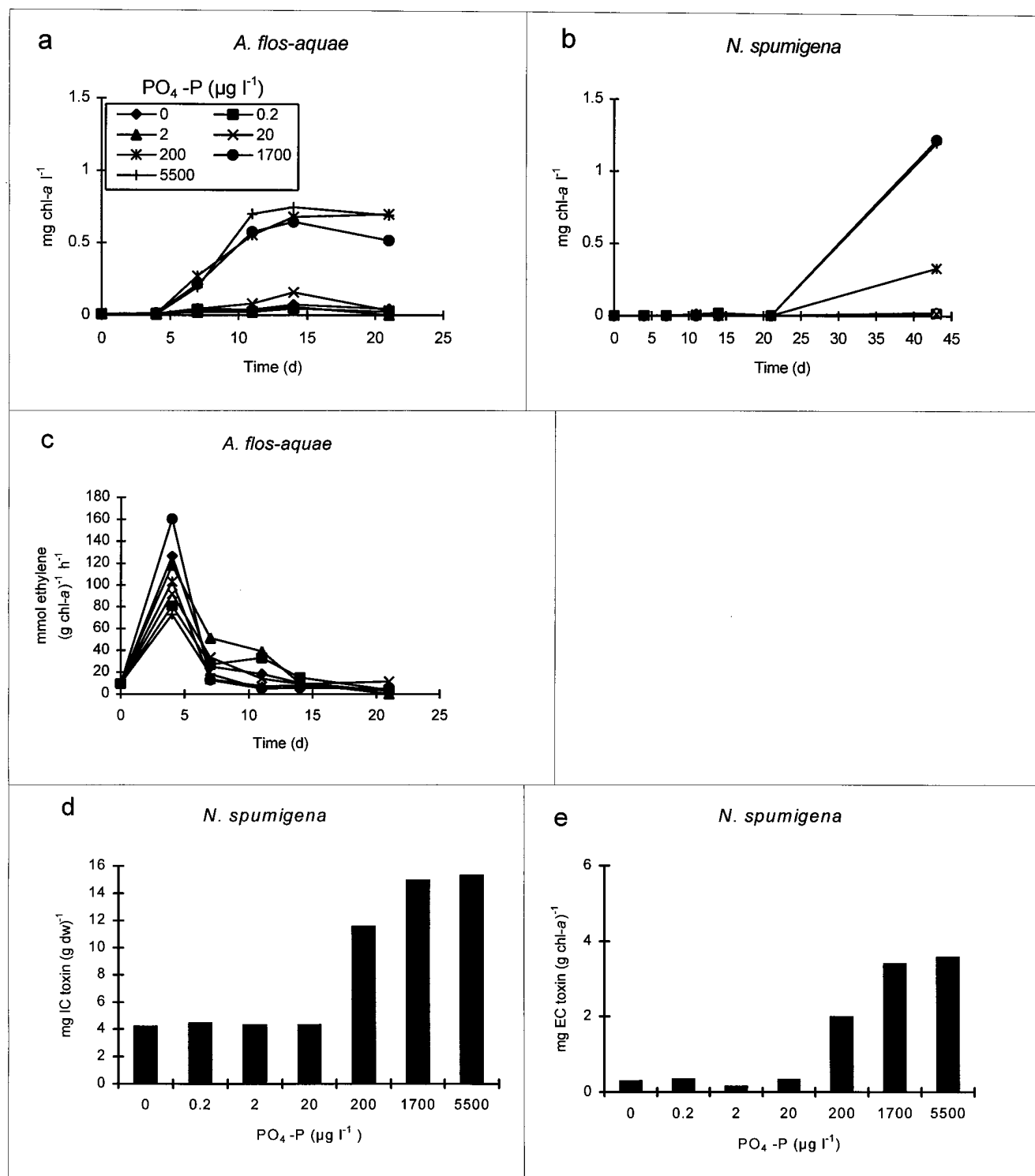Nitrogen fixation rate was often, but not always, highest

FIG. 5. Effect of phosphate on the biomass (a and b) and nitrogen fixation rate (c) of *A. flos-aquae* and *N. spumigena* cultures and on intracellular (IC) (d) and extracellular (EC) (e) toxin concentrations (toxin concentrations are average values of the replicates on day 43). Percent coefficient of variation was generally ≤30 in panels a to c, but in the cultures with poor growth it increased.

under conditions which promoted growth and lowest in cultures with poor growth. The highest nitrogen fixation rates were seen at the beginning of the exponential growth phase, and usually the nitrogen fixation rate decreased towards the

stationary growth phase. The decrease in nitrogen fixation rate in the course of long-term experiments, with the time scale of days, is usual (11, 34) and may be due to physiological changes in the cultures as they age. Therefore, as the cells reach sta-

tionary phase, the effect of treatment on the nitrogen fixation rate may be overridden by the effect of time (see phosphorus experiment for *A. flos-aquae* and bacteria and salinity experiment for both species). Because heterocysts are the primary location for nitrogenase, the frequency of these specialized cells is usually indicative of nitrogen fixation activity level in cyanobacterial filaments (9, 18). In this study, heterocyst frequency and nitrogen fixation rate of *A. flos-aquae* were positively related in many experiments. Moreover, the decrease in filament length of *A. flos-aquae* may indicate a decrease in its nitrogen fixation rate. In spite of the lower heterocyst frequency in *A. flos-aquae* than in *N. spumigena* (references 18, 22, and 25 and this study), the nitrogen fixation rate was generally higher in *A. flos-aquae* than in *N. spumigena*. These observations indicate differences in nitrogen fixation potential in heterocysts of these two species or distribution of nitrogenase in vegetative cells in *A. flos-aquae.*

The positive impact of accompanying bacteria on the growth and nitrogen fixation rate of cyanobacteria was previously shown for *Anabaena cylindrica* (19). In this study, only growth was promoted by accompanying bacteria. In the presence of heterotrophic bacteria, cyanobacteria may overcome oxygen concentrations inhibiting nitrogenase or carbon concentrations limiting photosynthesis (26). The possibility that the accompanying bacteria in our experiment were nitrogen fixers cannot be ruled out.

The factors favoring growth and nitrogen fixation generally increased toxin concentrations in the cells of *N. spumigena*. In this study, the optimum salinity for toxin production of *N. spumigena* was similar to that for growth. In previous studies, temperature and light flux have been shown to control toxin production in many cyanobacteria, such as *Anabaena* (27, 28), *Microcystis* (5, 39), *Nodularia* (17), and *Oscillatoria* (35). The same was true in this study, in which the highest toxin concentrations in *N. spumigena* were found at highest studied irradiances and temperatures. In addition, high phosphate concentration seems to increase the toxin production of *N. spumigena* (reference 17 and this study). These results are in line with those from studies with hepatotoxic *Anabaena* (28), *Microcystis* (16), and *Oscillatoria* (35). On the other hand, the neurotoxin (anatoxin a) production of *Anabaena* and *Aphanizomenon* was not altered by phosphate, although the lowest concentrations limited their growth (27). The negative effect of nitrogen on the nodularin production in *N. spumigena* was seen only in the highest nitrogen concentration, the same concentration which inhibited its growth and nitrogen fixation rate. Previously, nitrogen has been shown to decrease toxin concentration in *Anabaena* and *Aphanizomenon* (27, 28), but it seemed to increase toxin production of nonheterocystous cyanobacteria (5, 35). The associated bacteria had no effect on toxin production of *Oscillatoria* (35) or *Nodularia* (this study). In some cases, the responses in intracellular toxin concentrations paralleled the responses in nitrogen fixation rates. This observation needs further study, because cyanobacterial toxins have been suggested to act like metal-complexing siderophores (12) and nitrogenase enzyme requires iron as an essential cofactor. Toxin production of *Microcystis aeruginosa* has been shown to be influenced by iron (reference 40 and references therein). Moreover, the toxin-producing *M. aeruginosa* strain had a more efficient iron uptake system than the strain that did not produce toxin (40).

The concentrations of nodularin in growth media increased during the study period, probably as a result of the lysis of the cells. Cyanobacterial toxins are known to remain inside the cells and to be released into the growth medium when the cells die (3). According to this study, growth in different tempera-

ture, light, salinity, and phosphorus conditions may have an effect on the release of nodularin.

Several environmental factors controlled the growth and nitrogen fixation rate of *A. flos-aquae* and *N. spumigena*, as well as the nodularin production of *N. spumigena*. The two species showed different responses to changes in salinity, irradiance, temperature, and phosphorus. The results give physiological evidence which may explain the spatial and temporal distribution of these species in the Baltic Sea environment. According to this study, high irradiance, high temperature, and high phosphorus concentration may promote toxic blooms of *N. spumigena* in the Baltic Sea.

## REFERENCES

1. **Atlas, R. M.** 1993. Tryptone glucose yeast agar, p. 840. *In* L. C. Parks (ed.), Handbook of microbial media. CRC Press, Inc., Boca Raton, Fla.
2. **Bell, R. T.** 1993. Estimating production of heterotrophic bacterioplankton via incorporation of tritiated thymidine, p. 495–502. *In* P. F. Kemp, B. F. Sherr, E. B. Sherr, and J. J. Cole (ed.), Handbook of methods in microbial ecology. Lewis Publishers, Boca Raton, Fla.
3. **Berg, K., O. M. Skulberg, and R. Skulberg.** 1987. Effects of decaying toxic blue-green algae on water quality—a laboratory study. Arch. Hydrobiol. **108:**549–563.
4. **Carmichael, W. W.** 1994. The toxins of cyanobacteria. Sci. Am. **270:**78–86.
5. **Codd, G. A., and G. K. Poon.** 1988. Cyanobacterial toxins. Proc. Phytochem. Soc. Eur. **28:**283–296.
6. **de Silva, E. D., D. E. Williams, and R. J. Andersen.** 1992. Motuporin, a potent protein phosphatase inhibitor isolated from the Papua New Guinea sponge *Theonella swinhoei* Gray. Tetrahedron Lett. **33:**1561–1564.
7. **Herbert, D., P. J. Phipps, and R. E. Strange.** 1971. Chemical analysis of microbial cells. Methods Microbiol. **5B:**209–344.
8. **Hobbie, J. E., R. J. Daley, and J. Jasper.** 1977. Use of Nuclepore filters for counting bacteria by fluorescence microscopy. Appl. Environ. Microbiol. **33:**1225–1228.
9. **Horne, A. J., J. C. Sandusky, and C. J. W. Carmiggelt.** 1979. Nitrogen fixation in Clear Lake, California. 3. Repetitive synoptic sampling of the spring *Aphanizomenon* blooms. Limnol. Oceanogr. **24:**316–328.
10. **Hübel, H., and M. Hübel.** 1980. Nitrogen fixation during blooms of *Nodularia* in coastal waters and backwaters of the Arkona Sea (Baltic Sea) in 1974. Int. Rev. Gesamten. Hydrobiol. **65:**793–808.
11. **Huber, A. L.** 1986. Nitrogen fixation by *Nodularia spumigena* Mertens (cyanobacteriaceae). 2: Laboratory studies. Hydrobiology **133:**193–202.
12. **Humble, A., G. M. Gadd, and G. A. Codd.** 1994. Polygraphic analysis of the interaction between cyanobacterial microcystin (hepatotoxin) variants and metal cations, abstr. 82b, p. 82. *In* G. Tedioli, S. Ventura, and D. Zannoni (ed.), VIII International Symposium on Phototrophic Prokaryotes.
13. **Kahru, M., U. Horstmann, and O. Rud.** 1994. Satellite detection of increased cyanobacteria blooms in the Baltic Sea: natural fluctuation or ecosystem change? Ambio **23:**469–472.
14. **Kononen, K., J. Kuparinen, K. Mäkelä, J. Laanemets, J. Pavelson, and S. Nômmann.** 1996. Initiation of cyanobacterial blooms in a frontal region at the entrance to the Gulf of Finland, Baltic Sea. Limnol. Oceanogr. **41:**98–112.
15. **Kononen, K., K. Sivonen, and J. Lehtimäki.** 1993. Toxicity of phytoplankton blooms in the Gulf of Finland and Gulf of Bothnia, Baltic Sea, p. 269–273. *In* T. J. Smayda and Y. Shimizu (ed.), Toxic phytoplankton blooms in the sea. Elsevier Science Publishers B.V., Amsterdam, The Netherlands.
16. **Kotak, B. G., A. K.-Y. Lam, E. E. Prepas, S. L. Kenefick, and S. E. Hrudey.** 1995. Variability of the hepatotoxin microcystin-LR in hypereutrophic drinking water lakes. J. Phycol. **31:**248–263.
17. **Lehtimäki, J., K. Sivonen, R. Luukkainen, and S. I. Niemelä.** 1994. The effects of incubation time, temperature, light, salinity, and phosphorus on growth and hepatotoxin production by *Nodularia* strains. Arch. Hydrobiol. **130:**269–282.
18. **Lindahl, G., K. Wallström, and G. Brattberg.** 1980. Short-term variations in nitrogen fixation in a coastal area of the Northern Baltic. Arch. Hydrobiol. **89:**88–100.
19. **Love, A. J. W., and D. M. Rawson.** 1986. A note on the effects of associated micro-organisms on the growth and nitrogenase activity of the cyanobacterium *Anabaena cylindrica*. J. Appl. Bacteriol. **60:**143–146.
20. **Melin, K. E. R., and P. E. B. Lindahl.** 1973. Algal biotest of Stockholm

archipelago waters—qualitative aspects. OIKOS Suppl. **15:**189–194.

21. **Meriluoto, J. A. O., and J. E. Eriksson.** 1988. Rapid analysis of peptide toxins in cyanobacteria. J. Chromatogr. **438:**93–99.

22. **Moisander, P., J. Lehtimäki, K. Sivonen, and K. Kononen.** 1996. Comparison of $^{15}N_2$ and acetylene reduction methods for the measurement of nitrogen fixation by Baltic Sea cyanobacteria. Phycologia **35:**140–146.

23. **Namikoshi, M., B. W. Choi, R. Sakai, F. Sun, K. L. Rinehart, W. W. Carmichael, W. R. Evans, P. Cruz, M. H. G. Munro, and J. W. Blunt.** 1994. New nodularins: a general method for structure assignment. J. Org. Chem. **59:** 2349–2357.

24. **Nehring, S.** 1993. Mortality of dogs associated with a mass development of *Nodularia spumigena* (cyanophyceae) in a brackish lake at the German North Sea coast. J. Plankton Res. **15:**867–872.

25. **Niemistö, L., I. Rinne, T. Melvasalo, and Å. Niemi.** 1989. Blue-green algae and their nitrogen fixation in the Baltic Sea in 1980, 1982 and 1984. Meri **17:**3–20.

26. **Paerl, H. W., and J. L. Pinckney.** 1996. A mini-review of microbial consortia: their roles in aquatic production and biochemical cycling. Microb. Ecol. **31:**225–247.

27. **Rapala, J., K. Sivonen, R. Luukkainen, and S. I. Niemelä.** 1993. Anatoxin-a concentration in *Anabaena* and *Aphanizomenon* under different environmental conditions and comparison of growth by toxic and non-toxic *Anabaena*-strains—a laboratory study. J. Appl. Phycol. **5:**581–591.

28. **Rapala, J., K. Sivonen, C. Lyra, and S. I. Niemelä.** Variation of microcystins, cyanobacterial hepatotoxins, in *Anabaena* as a function of growth stimuli. Submitted for publication.

29. **Rinehart, K. L., K.-I. Harada, M. Namikoshi, C. Chen, C. A. Harvis, M. H. G. Munro, J. W. Blunt, P. E. Mulligan, V. R. Beasley, A. M. Dahlem, and W. W. Carmichael.** 1988. Nodularin, microcystin, and the configuration of Adda. J. Am. Chem. Soc. **110:**8557–8558.

30. **Rinehart, K. L., M. Namikoshi, and B. W. Choi.** 1994. Structure and bio-synthesis of toxins from blue-green algae (cyanobacteria). J. Appl. Phycol. **6:**159–176.

31. **Rinne, I., and E. Tarkiainen.** 1978. Algal tests used to study the chemical factors regulating the growth of planktonic algae in the Helsinki sea area. Mitt. Int. Ver. Limnol. **21:**527–546.

32. **Rouhiainen, L., K. Sivonen, W. J. Buikema, and R. Haselkorn.** 1995. Characterization of toxin-producing cyanobacteria by using an oligonucleotide probe containing a tandemly repeated heptamer. J. Bacteriol. **177:**6021–6026.

33. **Runnegar, M. T. C., A. R. B. Jackson, and I. R. Falconer.** 1988. Toxicity of the cyanobacterium *Nodularia spumigena* Mertens. Toxicon **26:**143–151.

34. **Sanz-Alférez, S., and F. F. del Campo.** 1994. Relationship between nitrogen fixation and nitrate metabolism in the *Nodularia* strains M1 and M2. Planta **194:**339–345.

35. **Sivonen, K.** 1990. Effects of light, temperature, nitrate, orthophosphate, and bacteria on growth of and hepatotoxin production by *Oscillatoria agardhii* strains. Appl. Environ. Microbiol. **56:**2658–2666.

36. **Sivonen, K., K. Kononen, W. W. Carmichael, A. M. Dahlem, K. L. Rinehart, J. Kiviranta, and S. I. Niemelä.** 1989. Occurrence of the hepatotoxic cyanobacterium *Nodularia spumigena* in the Baltic Sea and structure of the toxin. Appl. Environ. Microbiol. **55:**1990–1995.

37. **Sivonen, K., K. Kononen, A.-L. Esala, and S. I. Niemelä.** 1989. Toxicity and isolation of the cyanobacterium *Nodularia spumigena* from the southern Baltic Sea in 1986. Hydrobiology **185:**3–8.

38. **Tandeau de Marsac, N., and J. Houmard.** 1988. Complementary chromatic adaptation: physiological conditions and action spectra. Methods Enzymol. **167:**318–328.

39. **Utkilen, H., and N. Gjølme.** 1992. Toxin production by *Microcystis aeruginosa* as a function of light in continuous cultures and its ecological significance. Appl. Environ. Microbiol. **58:**1321–1325.

40. **Utkilen, H., and N. Gjølme.** 1995. Iron-stimulated toxin production in *Microcystis aeruginosa*. Appl. Environ. Microbiol. **61:**797–800.